FROM THE INSTITUTE OF BIOCHEMISTRY, UNIVERSITY OF UPPSALA, UPPSALA, SWEDEN

The Role of Metal Ions in the Catalytic Action of Peptidases

Studies on Carnosinase from Swine Kidney

by Andreas Rosenberg

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ANDREAS ROSENBERG

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1. Introduction

1.1. *Historical Background of the Problem*

Enzymes capable of catalyzing the hydrolysis of the peptide bond are termed proteolytic enzymes or peptidases. These enzymes are characterized by a remarkable specificity towards substrates of the same nature. Already in 1929, Linderstrøm-Lang (1) showed that the splitting of L-alanylglycine and L-leucylglycine by extracts from swine intestinal mucosa was due to two separate enzymes. Intense investigations during the following years proved that this group of enzymes was an excellent material for studies of the specificity of enzyme action, and much of the basic work on this problem by Waldschmidt-Leitz, Grassmann and Bergmann was done with peptidases.

Up to 1936 there was no evidence that peptidases require any kind of cofactor, but in this year Johnson *et al.* (2) discovered that the splitting of leucyl peptides by dialyzed "erepsin" was greatly enhanced by the addition of Mg^{++}. Since the observations b y these investigators the peptidase activity of a large number of tissue extracts has been shown to be influenced by metal ions. This resulted in a classification of peptidases as metal-ion activated and as enzymes unaffected by the presence of metal ions in solutions. This difference is rather fictitious since, as emphasized by Williams (3), all cations compete with protons in binding to basic groups and should thus always influence reactions where any of the partaking groups are of basic nature and available for proton binding. In the case of proteins and peptides this is naturally the rule. Anyhow, the magnitude and kind of the influence was so different for some of the enzymes that the term metal-activated had an operational justification. The phenomenon of metal-ion activation is not limited to peptidases, and a great number of different enzymes are classified as metal activated (4). Later a further classification of the metal-ion effects in peptidases had to be introduced, since carboxypeptidase, that was not classified among the metal-activated enzymes, was found to contain a firmly bound metal ion. The difference between the operational concepts for metalloenzymes, such as carboxypeptidase, and metal-activated enzymes has recently been discussed in connection with reviews of advances in studies of metalloenzymes (5) and metal-activated enzymes (6) and will not be discussed further in this connection.

In 1949, Smith (7, 8) forwarded a hypothesis attempting to explain the role of the metal ion in the mechanism of peptidase action. This hypothesis is in its main features

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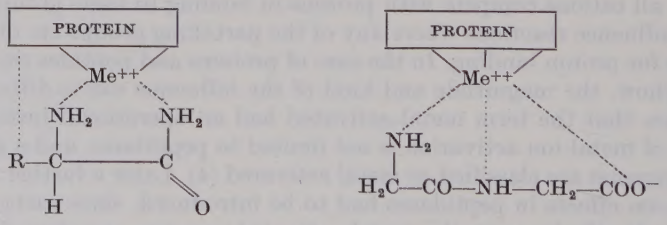
very similar to the model for arginase action proposed by Hellerman and Perkins (9). The work of Smith *et al.* was, as a rule, done with partially purified extracts from tissues and has often been criticized on these grounds. Later Smith *et al.* succeeded in preparing highly purified leucine aminopeptidase and repeated most of the previous experiments. The results confirmed his previous arguments and with a very slight modification Smith restated his theory (10). In this form, the Smith–Hellerman hypothesis is the only explanation of the metal-ion activation presented, that is based on experimental work with enzymes.

Since the main aim of the present work was to obtain more evidence about the mode of enzyme action in connection with investigations on the structure and function of metal enzymes, the Smith–Hellerman hypothesis formed a starting point and will be presented in detail in the next section together with the most relevant criticisms.

1.2. *The Smith–Hellerman Hypothesis and its Experimental Background*

Concisely stated, the hypothesis rests on the following postulates. The enzyme action takes place through the formation of a ternary enzyme–metal–substrate complex. At least two of the bonds between the enzyme and the substrate are via the metal ion. In addition to these bonds the substrate is bound by the side chain R to the enzyme. The two groups of the substrate, bound to the metal ion, are situated on either side of the susceptible peptide bond. In the case of leucine aminopeptidase these three bonds are: the hydrophobic bond between the aliphatic side chain of leucine and the enzyme, the bond between the free amino group and the metal ion and the bond between the nitrogen atom of the peptide linkage and the metal ion. The bonding via R and NH_2 is the initial and rate-determining step. After the formation of the third bond an immediate electron displacement takes place at the peptide link which results in a nucleophilic displacement at the electropositive carbonyl carbon. The amino-acids formed are rapidly displaced from the active site by the considerably higher proton affinity of the NH_2 group of the free amino-acid.

It follows from the hypothesis that the substrate specificity is largely determined by the nature of the R group and the pK value of the amino-acid formed. Further, differences between aminopeptidases and dipeptidases are explained by formation of two types of chelates: A, in the case of aminopeptidases and B, in the case of dipeptidases.



A clear implication of this kind of model is that the metal ion is essential and no enzyme action can take place in the absence of the necessary divalent ion.

It is desirable to examine the evidence available to support the hypothesis outlined above, together with the criticisms presented by different authors, since the nature

of both has largely determined the scope and aim of the present investigation. To begin with, the indispensability of the metal ion in the enzyme action rests on the fact that all the metal-ion activated peptidases are inhibited by complexing and chelating agents. Thus, a 10-fold excess of EDTA (ethylenediaminetetraacetic acid) reduces the activity of leucine aminopeptidase to 0.1 % of the initial value (10). The activity can be considerably reduced even by dialysis and a part of it restored after the addition of metal ions. The main criticism of experiments of this type is that the dialysis is followed by such high losses of activity that the restored activity is not very much higher than the activity of the enzyme preparation before any addition of metal ions.

The activity before the addition of metal ions or residual activity after the dialysis is, as a rule, so high that an enzymic catalysis in the absence of metal ion must be considered as possible. The inhibition of activity due to EDTA might be due to direct inhibition of the enzyme.

This point of view is most explicitly stated by Williams (11) who assumes that metal ions and protons function as Lewis acids and thus divalent ions only give a rather nonspecific, relative increase in the rate of the reaction. Despite this criticism, it seems that the indispensability of the metal ion is well supported by extensive investigations. A more serious criticism is that, although the experiments show that the metal ion is indispensable for the catalytic reaction, the experimental results can be adequately explained by the phenomenon of metal-ion stabilization of the enzyme, since in no case has it been shown that the inhibited enzyme can be reactivated after the activity measurement at 40° and after the removal of the chelating agent.

The evidence of the formation of a chelate between the metal ion and the enzyme is mainly based on the existence of a lag period in the metal-ion activation. Although it has been pointed out (12) that the combination of the metal ion with the protein cannot be responsible for the slow process of activation, the process can probably represent a subsequent rearrangement of the metal-ion–protein complex in which case it would still be an indication for an initial combination of metal ions with the protein. A more important objection is that the length of the lag period is a function of enzyme purity. This may indicate that the phenomenon need not be connected with the process of enzymic reaction. Furthermore, the qualitative spectroscopic evidence (13) for a chelate formation with substrate is of a dubious nature and probably due to secondary effects (14).

The principal evidence in support of the Smith–Hellerman hypothesis is the demonstration that the activity is a function of metal-ion concentration according to a simple mass-equilibrium equation. The only objection is that this does not constitute a proof of the nature of the interaction. A combination of the substrate with the metal ion to form a metallosubstrate or a combination of the metal ion with the enzyme in the role of a stabilizer would yield the same type of curves.

The structure of the chelate formed between the substrate and the metal ion and the resulting "electron pull" from the peptide bond are pure speculations, since there is no evidence presented or available about this type of metal chelates. For leucinamide, for example, there are no complex constants determined or any information published about the structure of the possible complex or chelate. The chelates postulated by Smith are criticized by Klotz (15) and Williams (16) who propose chelate structures of different type. Their suggestions and Smith's chelates are criticized by Rabin (17) who prefers his own brand of structures. These discussions furnish no new experimental data and must be considered as of little significance.

1.3. *The Scope of the Present Work*

A close scrutiny of the experimental data on peptidases revealed that very little additional information could be found from the previous studies or from studies of the same type in general. Additional data of another type were considered to be necessary.

This investigation was planned to include studies of activation and stabilization of a carefully purified peptidase, together with studies of the interaction of the metal ions with the substrate and the nature of the complexes and chelates formed. The experiments were designed in such a way that the objections presented in the previous section should be avoided as completely as possible.

The enzyme carnosinase was chosen for several reasons. Firstly, it showed all the properties considered to be characteristic of the whole group of enzymes, such as a lag period, narrow metal-ion specificity and instability of the enzyme. The most weighty argument was, however, the fact that carnosine was not hydrolyzed by any other known peptidase. This is of great importance since in studies of peptidases, with simpler dipeptides as substrates, a part of the activity measured must be ascribed to the presence of traces of other peptidases. This influences the determination of the residual activity, metal-ion specificity and practically every other measurement connected with activity determinations. Thus, the measured activity with carnosine as a substrate has a much better chance of being due to a single enzyme.

2. The Influence of Metal Ions on the Action of Carnosinase from Swine Kidney

2.1. *Materials and Methods*

Purification of the enzyme. The purification of the enzyme from swine kidney was accomplished by a procedure involving ammonium sulfate and acetone fractionation and zone electrophoresis on cellulose-stabilized columns (18). The main difficulty encountered in the purification was the narrow range of stability. At pH values lower than 7, rapid inactivation took place. In the region of 7.25–7.70, in the presence of Mn^{++} ions, the enzyme solutions were remarkably stable and could be stored for weeks at room temperature, without any significant change in activity. At pH values higher than 8, oxidation of the Mn^{++} ion resulted in coloured suspensions and, finally, in brown precipitates. The losses of activity must be ascribed to co-precipitation of the protein, since no loss of activity could be detected as long as the alkaline solution remained clear. The unfavourable conditions for stability are likewise the reason for the insufficient criteria on the physical properties of the enzyme. Electrophoresis can be performed only in the region if stability, i.e. in the immediate neighbourhood of the isoelectric point of the protein. Since the last step of purification involves prolonged electrophoresis at this pH, electrophoresis is not very sensitive as a criterion of homogeneity. Sedimentation measurements showed that the sensitivity of this method was not very good either, as the last steps of purification, representing a 50 % increase in specific activity, had very little influence on the sedimentation pattern. The apparent insensitivity may, of course, be due to the fact that the main part of the inactive material separated during the last steps of purification consisted of denaturated carnosinase. Because of the instability of the protein (19), it is possible that the pure enzyme obtained still contains a relatively large amount of inactivated

enzyme. Owing to these difficulties, it was considered as advisable to postpone a more detailed study of the physical properties of the enzyme until the process of stabilization was elucidated.

From the sedimentation data, the s_{20} could be estimated approximately as 5.5 (0.05 *M* veronal buffer, pH 8.05, 0.05 *M* NaCl, ~0.3 % protein).

Determinations of carnosinase activity. The enzymic activity was determined by titration of aliquots withdrawn from the assay solution. The procedure for titrations in ethanol, according to Grassmann, has been described by Smith (20). The use of a titration method excluded the possibility for a substantial variation of the substrate concentration, which was 0.05 *M* in all the activity measurements. A photoelectric method was used to determine the endpoint of titration. The activity was expressed as *a* moles carnosine split per minute, estimated from the linear part of the progress curve of the hydrolysis. In the case of measurements at low concentrations of Mn^{++} this is difficult, and the measured *a* represents the mean value for the period of measurement.

Analytical methods to determine the metal-ion concentration. The total Mn content of solutions was determined colorimetrically (21). The concentration of free Mn^{++} ions was estimated by measurements of electron-spin resonance of the free metal ions. The method had been described previously (22). The metal-indicator method of Raaflaub (23) was used to determine the concentrations of free Cd^{++} and Zn^{++} ions.

The content of different metal impurities in the protein solution was not determined, owing to experimental difficulties. This omission was not considered as very important since in the case of metal-activated enzymes the concentration of activating ions can be directly correlated to the activity. The presence of a constant, stoichiometric amount of a firmly bound metal ion indicates in this case only the existence of a site with strong chelating properties which has no connection with the phenomenon of metal-ion activation even if it can be an integral part of the protein structure.

Determination of the interaction of carnosine with the activating metal ions. The interaction with Cd^{++} and Zn^{++} was determined by potentiometric titrations according to the standard procedure (24). The interaction of Mn^{++} ions with carnosine was determined by measurements of the free metal-ion concentration in solutions of varying carnosine content. These measurements were carried out at a constant ionic strength of 0.16, to avoid changes which influenced the measurements of ESR. The values of formation constants could in this case be compared with the constants for other metal ions described in the literature. The presence of 0.16 *M* KNO_3 causes a difference in conditions between activity determinations and measurements of formation constants. This could not be avoided as measurements of activity in 0.16 *M* KNO_3 were hampered by the rapid oxidation of Mn^{++}. The metal-ion–carnosine interaction was determined, parallel to the methods described, also by the metal-indicator method. The results showed, in the case of Cd^{++}, an excellent agreement but in the case of Zn^{++} ion, a considerable discrepancy could be observed (25).

The determination of formation constants can under favourable conditions give us information about the total affinity of the metal ion for the substrate molecule and the nature of the groups involved in complex or chelate formation. It does not give any clue to the steric arrangement around the central ion and the influence of the metal ion on the electron distribution of the ligand molecule. Both of these properties are involved in the Smith–Hellerman hypothesis of a ternary complex. Methods suitable for investigations of this type of problem are, among others, X-ray crystallography and to a certain extent infra-red spectroscopy. Owing to the time-consuming

nature of X-ray work, only IR spectroscopy was used to study the influence of the metal ion on the peptide bond (26). The method giving the best results in this type of work was a development of the KBr disc procedure of Schiedt (27), suitable for studies at the temperature of liquid air. The method and its applications have been described previously (28).

Methods of removing the metal ions from enzyme solutions. Due to extensive and rapid denaturation of the metal-ion-free enzyme (19), dialysis was not suitable for removal of the metal ions. A procedure of selective electrodialysis at constant pH and constant ionic strength was developed and found suitable for this purpose (22). The electrodialysis was run near the isoelectric point of the enzyme which prevented precipitation on the membranes.

Materials. The substrate L-carnosine was either synthetized (18), or a commercial, chromatographically pure, preparation from Schuchardt was used. For preparations of buffers and metal-ion solutions only analytical grade reagents were used. Buffer and substrate solutions were purified by extraction with dithizone. Distilled water passed through a mixed-bed ion exchanger was used for all purposes. The glassware and pipettes were cleaned with a 1:1 mixture of concentrated sulfuric and nitric acids. Polyethylene containers were used to store the different solutions.

2.2. *The Role of Metal Ions in Carnosinase Catalyzed Reactions*

One of the most common objections to generalisations, based on studies of metal-ion activated enzymes, is that complete removal of metal ions is not followed by complete abolition of activity (29). The presence of residual activity is often claimed to prove that the enzyme can function even in the absence of activating ions, since protons can perform the same task as the metal ions. The question of the origin of the residual activity has been thoroughly discussed and the conclusion is that a small residual activity can scarcely be avoided without the presence of chelating agents (30).

The residual activity is not confined only to metal-activated enzymes. The persistence of residual activity is nicely illustrated by recent studies by Vallee *et al.* (31), where the experiments with removal of metal ions from carboxypeptidase always gave relatively high residual activities, although in the case of carboxypeptidase very strict studies by Vallee conclusively showed the metal ion to be an absolute requirement for activity.

Although the existence of a small residual activity is thus not evidence for the nonspecific nature of the metal-ion activation, even this assumption is a question of degree and a residual activity of 20 % of the original activity can by no means be called a small residual activity. The question of the possibility of catalysis without the presence of divalent ions is rather crucial for peptidases since the Smith-Hellerman hypothesis involves chelate formation, in which case divalent ions can scarcely be replaced by Na^+, K^+ or H^+. In the case of peptidases, the residual activities of the enzymes are high and the only experiment of significance is the study of purified leucine aminopeptidase. Here a tenfold excess of EDTA reduces the residual activity to 0.1 % of the initial value. The use of EDTA can naturally be objected to on the ground that EDTA may inhibit the enzyme by direct binding. This is to be considered as rather improbable, becuase it would be a curious coincidence that all metal-ion activated enzymes of different types should always be inhibited by all chelating agents without the metal ion being involved. A much more grave objection to the studies of leucine aminopeptidase is the fact that in no case is it shown that the enzyme solu-

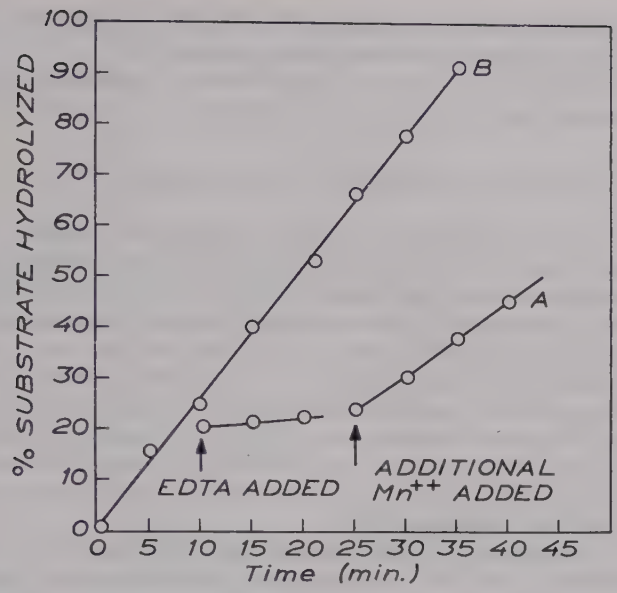


Fig. 1. Hydrolysis of carnosine at pH_{40} 7.65. Initial concentration of Mn^{2+}, 2×10^{-3} *M*. Curve A represents the activity after successive addition of EDTA and Mn^{2+}.

tion inhibited by EDTA could be reactivated after the removal of EDTA. On the contrary, this seems rather improbable since already dialysis against EDTA results in heavy losses of activity.

It is reasonable that in inhibition studies the bulk of the enzyme, after the addition of EDTA, should be denatured during the incubation at 40° and before the beginning of the activity determination. Nothing in these experiments prevents us from ascribing the entire apparent activation by metal ions to a case of metal-ion stabilization of the enzyme against proteolysis or thermal denaturation.

Consequently the first studies with carnosinase were directed toward an unequivocal determination of the role of the metal ion. The first experiments showed (22) that electrodialysis was followed by a fast decrease of activity, which could be restored by addition of Mn^{++} ions. The residual activity without any kind of chelating agents was approximately 5 % of the initial value. An addition of EDTA resulted in a residual activity which was too low to be measured. The removal of EDTA by prolonged electrodialysis resulted in a residual activity of approximately 2–6 %. It is possible that prolonged dialysis could further reduce the residual activity but the instability of metal-ion-free enzyme makes such experiments rather senseless. The activity measurements were performed both at 40° and 10° and the consistent results indicate that the reversible loss of activity after removal of Mn^{++} ions is not solely due to stabilizing effects of the metal ion.

The activating role of the metal ion is more clearly indicated by another experiment where the enzymic hydrolysis in the presence of Mn^{++} was interrupted by addition of EDTA. After 15 minutes an excess of Mn^{++} was added to restore the conditions before the addition of EDTA (Fig. 1). The difference in the initial and final velocities

indicates that, besides activating the enzyme, Mn^{++} also stabilizes the enzyme, a conclusion verified by stabilization studies on the enzyme (19).

It was quite clear from the above that the two functions, activation and stabilization, were both dependent on the presence of metal ions.

2.3. *The Stoichiometry of the Activation*

The next point of criticism of studies on metal-ion activation is based on the fact that the activity as a function of metal-ion concentration does not reach a limiting value according to a normal mass-equilibrium equation (29). This objection is scarcely warranted for studies of peptidases, as the logarithmic curves of activity versus metal-ion concentration (10) can easily be plotted in the form of Lineweaver-Burk plots (32), the resulting straight lines (33) indicating a 1:1 stoichiometry for each active site. Thus, the stoichiometry of metal-ion activation rests on the same type of kinetic evidence as the whole Michaelis–Menten theory. The only objection to the studies of Smith *et al.* is the fact that the metal-ion concentration represents the total metal-ion concentration and not the free metal-ion concentration. This leads to an error in the estimation of the kinetic constants (34).

In the case of Mn^{++} activation of leucine aminopeptidase the correction is negligible, since ESR measurements of free Mn^{++} concentration in the presence of leucineamide and THAM buffer of the pH of Smith's measurements, in this laboratory, showed that the influence of leucineamide on Mn^{++} concentration was negligible. The total Mn values of Smith actually represent the free metal-ion concentration with an error of a few percent. The binding to the substrate was much more predominant in the case of carnosine, especially in the case of metal ions such as Cd^{++} or Zn^{++}. In order to avoid gross errors, the free metal-ion concentration was determined for the mixtures of substrate and buffer. Calculations with the association constants, determined separately, agree well with direct measurements in the case of Mn^{++} and Cd^{++}. Zn^{++} values show a discrepancy between the methods of analysis (25). The stoichiometry of Zn^{++} activation is not discussed in the absence of more definite data about the real free metal-ion concentration. The enzymic activity (a) was determined as a function of the concentration (M) of free Mn^{++} and Cd^{++} ions (22). When an attempt was made to interpret the results in form of the simple Warburg–Christian relationship

$$a = \frac{a_{\max} M}{K_a + M}, \tag{1}$$

the reciprocal equation

$$\frac{1}{a} = \frac{K_a}{a_{\max}} \frac{1}{M} + \frac{1}{a_{\max}} \tag{2}$$

did not yield straight lines for Mn^{++} activation at higher temperatures. The Mn^{++} activation at low temperatures and Cd^{++} activation from 0° to 23° could adequately be described by the above equations (Fig. 2).

The equations above are nothing more than a very general form of describing the combination of two components in a 1:1 compound according to the mass-equilibrium law. They are formally identical with the equations for enzyme–substrate kinetics of Michaelis–Menten (32) and imply that there is a one-to-one stoichiometry for each active site. The divergence from the predicted curves at higher temperatures, in the

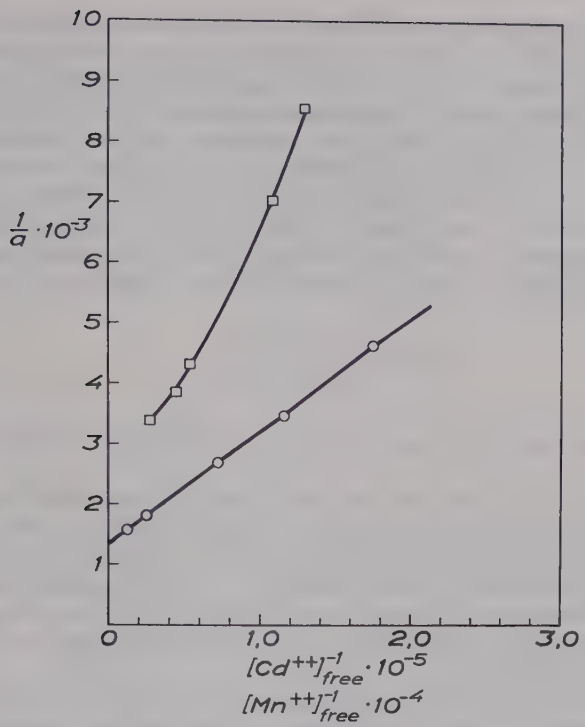


Fig. 2. Lineweaver–Burk plots (Eq. 2) for the activation of carnosinase by: □ – □ –, Mn^{2+} at 30°, pH 7.65 and ○ – ○, Cd^{2+} at 12°, pH 7.65.

presence of Mn^{++} ions, could naturally be explained by experimental error due to the difficulties of activity measurements, but if the constants calculated from different experimental points at three different temperatures are compared, there emerges a general tendency that is definitely outside the error of measurement (Table I).

An upward curvature of the reciprocal plot (Eq. 2) was predicted by Friedenwald and Maengwyn-Davies (35) for enzymic reactions where the substrate independently functions as an activator.

Table I. The kinetic constants (Eq. 2) for Mn^{++} activation of carnosinase calculated from different experimental points.

Temp . . . 40°		30°		20°	
$[Mn^{++}]_{free}$	K_a	$[Mn^{++}]_{free}$	K_a	$[Mn^{++}]_{free}$	K_a
3.3×10^{-5}	6.2×10^{-5}	6.5×10^{-5}	1.4×10^{-4}	6.5×10^{-5}	3.0×10^{-4}
6.5×10^{-5}	5.1×10^{-5}	9.1×10^{-5}	1.2×10^{-4}	9.1×10^{-5}	2.8×10^{-4}
9.1×10^{-5}	4.0×10^{-5}	1.9×10^{-4}	0.75×10^{-4}	2.0×10^{-4}	1.6×10^{-4}
2.0×10^{-4}	2.5×10^{-5}	2.3×10^{-4}	0.48×10^{-4}	4.0×10^{-4}	0.8×10^{-4}

In the case of carnosinase the two independent functions are assumed to be activation and stabilization. The assignment based on kinetic data was only tentative. Separate studies of stabilization (19) showed that stabilization by Mn^{++} ions really took place in the concentration range for metal-ion activation. No efforts have been made to compare the kinetic constants from Mn^{++} activation and Mn^{++} stabilization, since in no case could the real initial velocities be determined with the necessary precision. For the purposes of the present discussion the demonstration of two functions in the same range of metal-ion concentration, both following a mass-equilibrium law, are more than adequate to explain the deviation of Mn^{++} activation curves from the form predicted by Equations 1 and 2. The activation curves can be described by the equation

$$\frac{a_{max}}{a} = 1 + \frac{K_1}{M} + \frac{K_2}{M^2}, \tag{3}$$

where K_1 and K_2 represent the kinetic constants for metal-ion activation and metal-ion stabilization.

Activation by Cd^{++} ions at the temperatures studied followed the simple Warburg–Christian equation (Eq. 1). Activation was not studied at higher temperatures than 21° but as Mn^{++} activation already at this temperature shows deviations from Equation 1, it would be reasonable to assume that Cd^{++} does not partake in the stabilization equilibrium. This does not exclude the possibility that Cd^{++} stabilizes the enzyme by the binding to the active site, in which case the kinetic equation is of the same kind as described by Equation 1.

Evidence discussed in this and the preceding section definitely establishes that the metal ion is indispensable for the enzymic reaction and that the process of activation is not identical with the process of stabilization. The kinetic measurements showed that there was a 1:1 stoichiometry at every active site.

Thus, on firm evidence, we can assert that metal ions in the case of carnosinase function as a coenzyme.

It would be of interest to compare the kinetics of activation of a number of metal-ion activated enzymes when they are activated by the same metal ion. Figure 3 shows the Mn^{++} activation of carnosinase (22), leucine aminopeptidase (10) and glycyl-L-leucine dipeptidase (36). In the case of the two first enzymes the metal-ion concentration represents the concentration of free metal ion. In the case of glycyl-L-leucine dipeptidase the values refer to the total concentration of the metal ion. The free metal-ion concentration and the approximate K_a value can be estimated from the formation constant of glycylglycine-Mn. Table III compares the magnitude of K_a of different metal-ion activated enzymes with the formation constants of the substrate-Mn

Table II. The kinetic constants for Cd^{++} activation of carnosinase at pH 7.65 and different temperatures.

Temp C°	K_a
21	5×10^{-6}
12	1.5×10^{-5}
0	1.2×10^{-4}

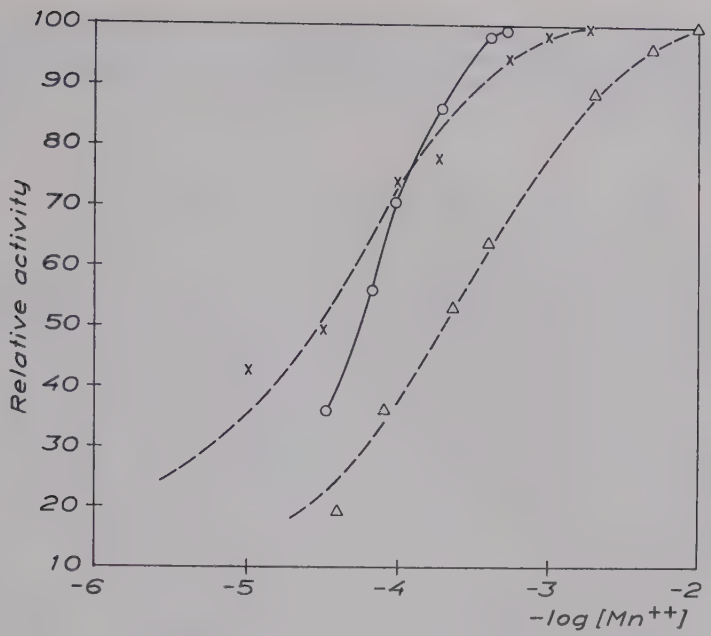


Fig. 3. Relative enzymic activity as a function of Mn^{++} concentration: ○ – ○ – ○ carnosinase, at 40°, pH 7.65; × – × × – × – leucine aminopeptidase, at 40°, pH 8.6; △ – △ – △ glycyl-L-leucine dipeptidase, at 40°, pH 7.6.

compounds. The interaction of leucineamide with Mn^{++} was investigated by ESR studies. No measurable effect could be detected in the concentration region suitable for analysis. The K_{eq} for glycyl-L-leucine is estimated from the constants determined for glycylglycine-Mn interaction (37). All the K_a values refer to the concentration of free metal ion. The K_a for enolase (38) has been determined at 20° but can be compared to the constants determined at 40°, since the K_a of enolase shows very little change with temperature (39).

The kinetic constants, K_a, for all peptidases and enolase are surprisingly similar; the Mn^{++} activation takes place in the region of $1–5 \times 10^{-5}$ M Mn^{++}. The K_a values for leucine aminopeptidase and enolase differ by a factor of 2, while the binding of the metal to substrate differs by a factor of 100. An obvious conclusion would be

Table III. The kinetic constants K_a (Eq. 1) for a number of Mn^{++}-activated enzymes.

Enzymes	Temp. C°	K_a	Substrate	log K_{eq}	Ref.
Carnosinase	40	4×10^{-5}	Carnosine	1.43	(22)
Glycylleucine dipeptidase	40	5×10^{-5}	Glycyl-L-leucine	2	(36)
Leucine aminopeptidase	40	3×10^{-5}	Leucineamide	0.5	(10)
Enolase	20	1.5×10^{-5}	Phosphoglyceric acid	2.3	(30)

that the binding of Mn^{++} to the enzymes takes place through the same kind of groups. Direct binding studies would be of great interest in this case. Such measurements are unfortunately difficult to perform with peptidases due to the instability of the metal-ion-free enzyme.

2.4. *The Nature of the Protein–Metal Interaction*

Of different possibilities for metal-ion–enzyme interaction, the Smith-Hellerman hypothesis favours the binding of metal ion by the formation of a chelate. This enzyme–metal chelate constitutes a part of the active site and is capable of binding substrate molecules. The main argument for the existence of such a step is the existence of a lag period in metal-ion activation. As previously mentioned in section 1.2, it has been pointed out that the slow step cannot represent the binding of metal to protein. This, however, does not invalidate the argument of Smith as, if the combination of metal ion with the enzyme is followed by a slow rearrangement of the enzyme molecule, the existence of a lag period furnishes strong evidence for the Smith-Hellerman hypothesis. The criticism by Rabin (17) that even the supposed enzyme rearrangement cannot account for the lag period, due to the principle of microscopic reversibility, cannot be accepted because the instantaneous loss of activity after the removal of metal ions has no connection with the equilibrium $E \rightleftharpoons E'$, as the lag period is determined by the equilibrium $EM \rightleftharpoons E'M$. Even if the equations should be identical, the reasoning by Rabin is only valid for $K_{eq} = 1$.

The metal-ion activation of the purified carnosinase was thoroughly and repeatedly investigated for the existence of a lag period, but none could be detected. This means that the combination of the purified metal-ion-free enzyme with the activating ions is not followed by any kind of slow rearrangement as far as the experimental method allows us to observe. The absence of the lag period naturally does not prove that chelate formation does not take place. In fact, chelate formation with the enzyme has been ascribed, with strong experimental support, to enzymes (30) where the lag period has never been observed. The absence of a lag period in purified carnosinase and the presence of a lag period for partially purified enzyme solutions would rather indicate that the lag period can be due to a slow rearrangement of partially denaturated enzyme. This is supported by the fact that the lag period diminishes with the degree of purity.

If the rest of the available experimental material is reviewed, there seems to be no other direct evidence for chelate formation between the metal ion and the enzyme.

Some of the observations on carnosinase might point towards chelate formation. For example, the temperature dependence of the Mn^{++} and Cd^{++} activation of carnosinase might be interpreted as evidence for chelate formation. This is supported by the fact that metal-ion–substrate interaction has an opposite temperature dependence. The observed temperature dependence could thus be due to the rearrangement of the protein molecule followed by an exceptionally high entropy change. The temperature dependence can be explained alternatively by assigning it to the substrate–enzyme interaction, since investigations of substrate–metal interaction showed that, in the pH region studied, the substrate has three charged groups ready for binding. A charge neutralisation would be expected to be strongly temperature dependent (40).

2.5. *The pH Dependence of the Metal-ion Activation*

The shift in pH-activity curves for different activating ions was first discovered and interpreted by Stock, Perkins and Hellerman (41). They attributed this difference to variations in stability of the complexes between metal ion and enzyme or metal ion and substrate and used these differences as a proof for the existence of competition between protons and metal ions in binding to the enzyme or substrate.

This interpretation is without doubt possible and probable. The shift of Cd^{++} activation of carnosinase towards lower pH values compared to the Mn^{++} activation curve (22) is in accordance with the stronger binding of Cd^{++} by the substrate (25). It is very interesting to note that the relative positions of the pH-activity curves are changed when glycyl-L-histidine is used as the substrate instead of carnosine (22). Changes in the free metal-ion concentration can, as judged from the plot activity–free metal-ion concentration, scarcely account for the effect. Neither can a change in the metal-ion independent binding of the substrate to the enzyme change the curves in such a selective way. These shifts in the pH-activity curves of carnosinase support that part of the Smith-Hellerman hypothesis which assumes that some kind of interaction between the metal ion and the substrate is involved in the catalytic process.

The pH dependence of metal-activated enzymes has been exploited more extensively by Roholt and Greenberg (42) who calculated the kinetic constants for a proposed arginase mechanism from such measurements. Such results are of dubious value as changes in the concentration of the activating ion as well as the hydrolysis of the metal ion at higher pH values (43) have been neglected; neither has the inactivation and its dependence on metal-ion concentration been accounted for. It must be stressed that the metal-ion activated enzymes cannot be treated according to the standard formulae for pH dependence of enzymes, as metal-ion activation involves competition between the metal ions and protons. If the kinetic constants are calculated, it is necessary to determine the pM as well as the pH of the solutions measured.

Owing to these complications and difficulties in measuring the true initial velocities, no further interpretation of such data has been considered as meaningful.

2.6. *The Kinetics of Inactivation of Carnosinase and the Metal-ion Stabilization of the Enzyme*

It was emphasized in the preceding section that no activation measurements can give relevant data on the metal-ion activation unless the possible stabilization of the enzyme molecule by the same ions is studied and accounted for. The preliminary studies of carnosinase-containing tissue homogenates by Hanson and Smith (44) indicated that carnosinase was stabilized by Mn^{++} ions. Studies of metal-ion stabilization showed that the metal-ion-free enzyme was extremely unstable, loosing about 80 % of its activity irreversibly during one hour at 40°. In the presence of Mn^{++} ions the enzyme was very stable and could be stored for weeks at room temperature. This implies that the metal-ion stabilization is an essential feature of this enzyme and that the stabilizing ions are as indispensable as the activating ions. It is evident that, if the stabilization is not due to some more general effect of ionic strength, the specificity of metal-ion stabilization should considerably influence the apparent metal-ion specificity of activation. Figure 4 demonstrates a difference in stabilizing properties between two ions activating the enzyme.

Studies of the irreversible inactivation of the metal-ion-free carnosinase (19) showed

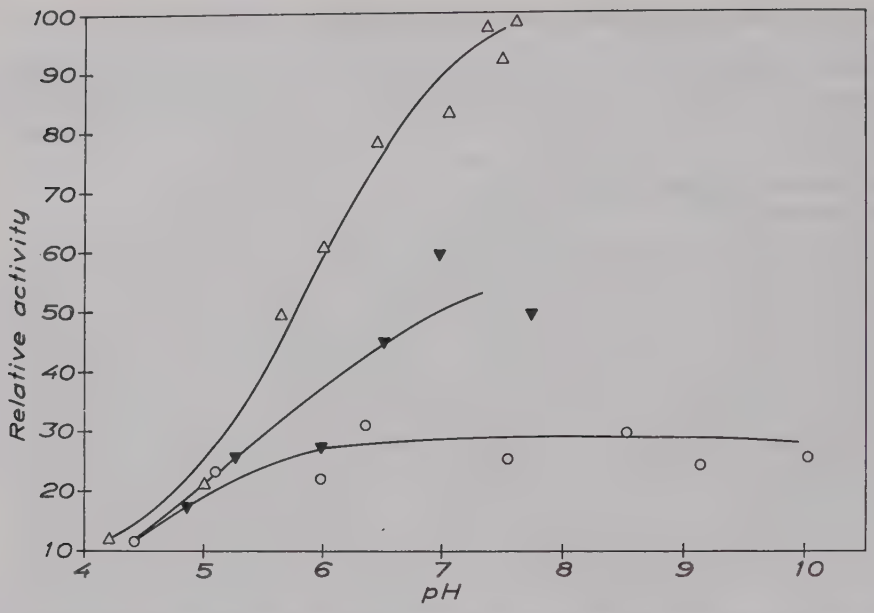


Fig. 4. The irreversible inactivation of carnosinase at 40° as a function of pH in presence of: △ – △ – △ $5 \times 10^{-3} M$ Mn^{++}, ▼ – ▼ – ▼ $5 \times 10^{-3} M$ Cd^{++}, and ○ – ○ – ○ metal-ion-free solution.

that the reaction was not of first order with respect to time. The semi-logarithmic plot of the inactivation showed a curve composed of two linear portions, one dominating the first part of the reaction and the other accounting for the latter part of the inactivation curve. The possibility of two different active components could be excluded as the intercept of the second linear part of the reaction curve was not indepedent of temperature as expected for a mixture of two enzymes.

It could be shown that the simplest scheme to account for the kinetics of irreversible inactivation was a modification of the mechanism proposed by Wright and Shomaker (45) for the denaturation of diphtheria antitoxin

$$\begin{array}{ccc} & H^+ & \\ EMn & \rightleftharpoons & E \rightleftarrows E' \\ & Mn^{++} & \searrow \swarrow \\ & & D \end{array} \qquad (4)$$

E and E′ are two forms of the enzyme and D the irreversibly denaturated part of it. This mechanism is represented by a velocity equation

$$R = Ae^{-\lambda_1 t} + Be^{-\lambda_2 t} \qquad (5)$$

where R represents the residual activity after reactivation with Mn^{++} ions. A, B, λ_1 and λ_2 are constants. The best possible curve through the experimental points could be represented by an equation of this type (19).

An important premise for the study of metal-ion stabilization is the knowledge that the inactivation is not due to proteolytic degradation of the enzyme by traces of proteolytic enzymes present as impurities. In the latter case the phenomenon of metal-ion stabilization can be due to inhibition of the proteolytic enzymes and has no connection with the structure and function of the enzyme studied. In the case of carnosinase, colorimetric determination of the free amino-acids, chromatography and paper electrophoresis failed to show any evidence of a breakdown of the enzyme molecules (19). On the contrary, the inactivation is followed by decreased solubility which indicates the presence of thermal denaturation. Unfortunately, decreased solubility prevents sedimentation and diffusion studies of the denaturated enzyme solutions.

The stoichiometry of the metal-ion stabilization is more difficult to determine. If the real initial velocities could be determined, the velocity of the inactivation should be proportional to the enzyme, free of the stabilizing metal ion. The resulting Lineweaver-Burk plot should, in this case, show if the stabilization is characterized by a simple 1:1 stoichiometry for each active site. The results of Mn^{++} stabilization of carnosinase can be plotted in such a fashion and a straight line drawn to fit the experimental points. The dispersion of the experimental values for residual activity is, however, of such a magnitude that an unequivocal determination of the stoichiometry of the stabilization is not feasible.

2.7. *The Nature of the Metal-ion Stabilization*

The main question about the nature of stabilization is connected with the possibility that the stabilization is a side-result of the metal ion combining with the enzyme as a coenzyme. In this case the stabilization equilibrium is identical with the activation equilibrium. On the other hand, stabilization may take place independently at a different site.

The most evident approach to this problem is, of course, the comparison of the metal-ion specificity of activation with the specificity of stabilization. Differences in these two specificities were rather obvious. While the relative efficiency of activation was given (22) by the series

$$Cd^{++} > Mn^{++} > Zn^{++} > Co^{++},$$

the stabilization of carnosinase (19) was characterized by the following order

$$Mn^{++} > Ca^{++} > Mg^{++} = Cd^{++} > Zn^{++}.$$

The failure of Cd^{++} and, especially, Zn^{++} to stabilize the enzyme may depend on additional interaction with groups of the protein, a rather obvious possibility due to the relatively high pH value. Of more consequence are the excellent stabilizing properties of Ca^{++}, an ion that shows no activating properties whatsoever. This gives strong support to the hypothesis of the existence of two different and independent sites. More conclusive evidence for this interpretation would be a demonstration that the stabilization by Ca^{++} ions is not competitively inhibitory to Mn^{++} activation of the enzyme. Otherwise, the assumption that Ca^{++} may be bound to the active site but fails to bind the substrate in a ternary compound, is a valid objection to the two-site hypothesis. The measurement of the stability as a function of $[Ca^{++}]$ reveals that Ca^{++} stabilization takes place at approximately 10^{-4} M. Since Mn^{++} activation takes place at approx. 10^{-5} M, an investigation is feasible. The experimental results were

plotted as v_0/v against [Ca^{++}] at constant Mn^{++} concentration. The best line through the experimental points at 3 different Mn^{++} concentrations is a non-competitive plot. This answers the question about the existence of a different site for stabilization positively. Even if it was assumed that the plot was entirely competitive, the differences for the three Mn^{++} concentrations being within the limits of experimental errors, the calculated inhibition constant would be $K_i = 1 \times 10^{-2}$ which implies that the inhibition takes place at a 100 times higher Ca^{++} concentration than the stabilization. This supports strongly the hypothesis of one or more independent sites of stabilization through binding of divalent ions to the enzyme molecule.

The specificity of the metal-ion stabilization is rather low as all divalent ions investigated (in addition to the published claims, also Ba^{++} and Ni^{++}) influenced the enzyme stability. At 40° the univalent ions (K^+, Na^+) showed no effect at all. At low temperatures a certain effect could be discovered even for univalent ions. The difference of magnitude is, however, such that it can be said that the stabilization is selective between mono- and divalent ions.

A theory about the chemical nature of such stabilization has been advanced by Ruegamer, who interprets the pH stability curves of coliphage (46) and arrives at the conclusion that the stabilization is proportional to Ca^{++} concentration and inversely proportional to the second power of H^+. Ruegamer considers this as evidence for chelate formation between Ca^{++} ions and two groups on the protein surface. It fits well for carnosinase as far as the selectivity for divalent ions is concerned.

The pH stability curve of Mn-carnosinase (Fig. 4) may, of course, also be plotted in the form of

$$\mathrm{pH} = A + B \cdot \log \frac{R}{R_0 - R}, \tag{6}$$

where R_0 is the initial activity and R the residual activity after one hour at 40°. The residual activity is in this case found to be proportional to $(H^+)^{-1.7}$. This is evidence of the same nature as Ruegemer's and it is valid evidence for the displacement of two H^+ ions by the Ca^{++} ion if the supposed displacement is the rate-limiting step in the inactivation process.

The general conclusion from stabilization studies is that the stabilization by divalent ions is undoubtedly due to an independent equilibrium between the metal ion and the enzyme molecule. The stabilization is not an additional property important at abnormally high temperatures or under long periods of storage, but an intrinsic property of this enzyme, since the metal-ion-free enzyme undergoes a rapid, irreversible denaturation even at room temperature or lower.

2.8. *Metal–ion Specificity of Activation*

The apparent specificity of carnosinase determined by Smith (44) was rather narrow, a feature characteristic of all peptidases investigated by Smith *et al.* The enzyme was reported to be activated exclusively by Mn^{++} and Zn^{++}. The activation of purified carnosinase was reinvestigated for a number of metal ions at different pH values and different concentrations of the activating metal ion. The results showed activation by Cd^{++}, Mn^{++}, Zn^{++} and Co^{++} ions. The apparent efficiency is given by the order $Cd^{++} > Mn^{++} > Zn^{++} > Co^{++}$.

The pH-activity curves for Mn^{++} and Zn^{++} activation are in very good agreement with the results by Hanson and Smith (44). Cd^{++}, on the contrary, was reported to inhibit carnosinase activity. The failure to observe Cd^{++} activation may be due to many reasons, the most obvious one is the inefficiency of Cd^{++} as a stabilizer. The incubation used by Smith certainly must have resulted in an extensive loss of activity.

The metal-ion specificity has been considered by Smith to be a characteristic of the enzyme and assumed to be independent of the nature of the substrate. This is not in accordance with the mechanism proposed, because, if the Smith-Hellerman hypothesis with the supposed ternary complex is true, then the metal-ion specificity should vary, as the side chains of peptides have a considerable influence on the formation constants of their metal complexes with different ions. This is especially true in the case of substrates possessing other polar groups than the amino and carboxyl groups. This aspect of the peptidase specificity has received scanty attention; the only studies of this problem have been published by Vescia (47), who found considerable differences of this kind in the specificity of leucine aminopeptidase. Unfortunately the substrates used are hydrolyzed readily even by other known peptidases from the same source, and Smith reported that further purification of the enzyme cancelled these effects. Carnosinase is far more suitable for this kind of experiment, since it is the only known metal-activated peptidase to act on carnosine. If the activity towards carnosine is compared with the activity towards glycyl-L-histidine, a different, relative metal-ion specificity is observed. The Mn^{++} activity curve remains unchanged but activation by Cd^{++} is reduced to half of the value for carnosine and activation by Zn^{++} is scarcely observable. The change in this direction is fortunate, as it excludes rather definitely the possible influence of traces of other enzymes on the hydrolysis of glycyl-L-histidine.

This confirms that the type of effects reported by Vescia and predicted from the Hellerman–Smith hypothesis really does exist.

In connection with the considerably broadened metal-ion specificity of carnosinase, the question arises whether the reported absolute or nearly absolute specificities of peptidases are real or only apparent, owing to the conditions of activity measurements. This question has gained more interest since Vallee *et al.* showed with carboxypeptidase (48) that the metal ion can be interchanged even in metalloenzymes. As emphasized by Williams (3), we must distinguish between the metal-ion specificity due to spatial and electronic requirements and the specificity induced by secondary effects.

As secondary effects we must consider the removal of activating ions from solution by competing ligands such as OH, the instability of the valency state of the metal and chelate formation between other groups on the enzyme or substrate. An additional effect of this kind is the metal-ion stabilization, which results in apparent high specificity if the activity measurements are carried out under unfavourable conditions. In general, even inefficient removal of the ions present, manifested in high residual activity, tends to conceal activation that is not higher than the residual activity. The apparent metal-ion specificity of carnosinase can to a considerable extent be accounted for on such secondary grounds. This means that a reinvestigation of the specificity of some other peptidase is necessary before the absolute specificities claimed can be accepted. In a functional sense, of course, the specificity induced by secondary effects is as real and exclusive as any other specificity, the importance being here that in this case we cannot draw any conclusions about the nature of the active site. The same kind of reasoning is valid also in the case of metal-ion inhibition, suitably illustrated

by the measurements of Cu^{++} inhibition of carnosinase (22). The experiments show that the presence of 1×10^{-3} *M* Cu^{++} has no effect on the activity of carnosinase. Despite this it would be an error to conclude that the active site has no affinity for Cu^{++}. In this case the Cu^{++} is very strongly bound to the substrate, which acts as a chelating agent. The only conclusion from this experiment is that the interaction of Cu^{++} with the active site must be considerably weaker than with the substrate.

2.9. *The Interaction of Metal Ions with the Substrate and the Question of Metal–ion-induced Electronic Rearrangement of the Peptide Bond*

The formation constants for carnosine–metal complexes are available for Cu^{++} (49, 50), Ni^{++}, Cd^{++}, Zn^{++} (50) and Mn^{++} (25). The log k_1 values are compiled in Table IV.

The interaction with carnosinase is surprisingly weak for Mn^{++} when compared with the values of log k_1 for glycine-Mn (37) and glycylglycine-Mn (37), both of which (3.44 and 2.56) lie considerably higher. Even the interaction of Cd^{++} with carnosine is of the same magnitude as the interaction with glycylglycine or other simple peptides (51) with only two polar groups. Thus, the presence of 3 groups, capable of bond formation, does not increase the affinity to the metal ion. This shows that chelate formation is not probable. Since the titration curves of carnosine nitrate in presence of Cd^{++} ions show a definite point of inflexion after the addition of one equivalent of alkali (50), it is obvious that in the pH range of carnosinase activity the metal ion interacts with a nitrogen of the imidazole ring and not with the amino group, which thus remains protonated. The interaction with the ionized carboxyl group cannot be determined directly but as the formation constants of carnosine complexes are not higher than those for imidazole complexes, the interaction with carboxyl must be negligible.

The group interacting with the Mn^{++} ion cannot be determined directly. The only two reasonable possibilities are, firstly, the carboxyl group, with a possible extra bond to the peptide group, and, secondly, the imidazole nitrogen. The second possibility must be considered as the more probable since the log k_1 for Mn-carnosine is of the same magnitude as for Mn-imidazole (52) and Mn-histidine (53) (1.65 and 1.75).

Thus, the dominating species of the substrate molecule at the pH of enzyme activity is the zwitterion with the metal ion attached to the imidazole group. In the case of glycyl-L-histidine the Cd^{++} and Zn^{++} compounds may involve chelate formation to

Table IV. The successive formation and ionization constants for L-carnosine with divalent ions at 25° and 0.16 ionic strength.

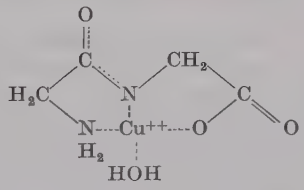
	Cu	Ni	Zn	Cd	Co	Mn
log k_1	4.2	2.80	2.30	2.50	2.25	1.4
log k_2		2.10	2.10	1.75	1.6	
log k_3		1.6	2.00			
log k_4			1.7			
pK_1	5.00	7.35		8.50		

the amino group (52). This may influence the apparent activating properties of Cd^{++} and Zn^{++} ions.

A separate question, in this connection, is the existence of electron pull from the peptide bond caused by the activating metal ion. A theoretical treatment of this problem by Goudot (54) shows that the rupture of the peptide bond is feasible if the metal ion interacts with the nitrogen of the peptide bond. From the viewpoint of the Smith–Hellerman hypothesis it would be of paramount importance to show that such interaction takes place and results in the rupture of the peptide link. The only indication in this direction has been the dissociation of an extra hydrogen from the peptide bond of the Cu-glycylglycine chelate (49), which has been interpreted as the substitution of the hydrogen by the metal ion. The method chosen for further investigations was the infra-red analysis of solid chelates of known composition. The peptides investigated were the Cu^{++}, Ni^{++} and Zn^{++} chelates of simple glycine dipeptides.

The results showed that in Cu^{++} chelates the metal ion interacts with the peptide nitrogen, which results in a considerable change in the electron distribution in the peptide link with the subsequent dissociation of the peptide hydrogen. In the case of Ni^{++} and Zn^{++} chelates isolated at the pH of the enzyme activity, the interaction with the peptide bond must be negligible, as the frequency changes were of the same magnitude as interactions between different molecules in the crystal.

The electron displacement in the peptide bond, as the result of interaction between Cu^{++} and nitrogen, does not, however, result in any measurable weakening of the peptide bond. Here we have a chelate formed according to the forms proposed by Smith and Hellerman



with the metal bound to both sides of the peptide bond and also to the peptide bond itself. In spite of this, Cu^{++} does not activate any of the known peptidases, neither does it exert any catalytic activity in the hydrolysis of the peptide bond. The structure of Cu-glycylglycine proposed from the infra-red studies has been confirmed by recent X-ray work (55). This implies that either the premises of Goudot's calculations are insufficient or that other effects, such as energy gains from ring formation more than compensate for the labilizing effect. Of the other metals investigated, Zn^{++} does activate peptidases. If there exists with these metal ions an interaction with the peptide bond through the oxygen or nitrogen, it must be of a relatively weak sort, and can scarcely account for such a decisive part in the catalytic mechanism as proposed by Smith. The predominant part of the catalytic properties must be ascribed to the protein part of the enzyme. Of course, IR studies of some chelates in the solid phase are not conclusive evidence against any possibility of interaction of the type proposed by Smith, but they render it rather improbable.

2.10. *The Mechanism of Metal-ion Activation*

The mechanism of metal-ion activation is for the most part assumed to follow one of the two possible pathways (6). The first alternative is characterized by an

initial binding of the metal ion to the enzyme molecule with a subsequent interaction of this complex with the substrate molecule:

$$E + M \rightleftharpoons EM + S \rightleftharpoons EMS. \quad (7)$$

This seems to be the case for enolase (30) and this is the alternative put forward for the peptidases by Smith (10). The second possibility is the initial combination of the metal ion with the substrate to form a metallo-substrate which is the real substrate for the enzyme. This explanation is shown to represent the enzymic reaction catalyzed by 3-phosphoglycerate-1-phosphokinase (PGK) in the presence of ATP and Mg^{++} (56).

$$M + S \rightleftharpoons MS + E \rightleftharpoons MSE. \quad (8)$$

The two alternatives presented are the two opposite extremities chosen to illustrate the point that even in the case of diametrically different mechanisms, kinetic data are often not sufficient to determine the pathway. The rate equations arrived at are similar, the only difference being that the kinetic constants have a different physical meaning (6). In cases where a definite pathway has been proposed this has been supported by measurement of independent parameters. Thus, in the case of enolase (30) and PGK (56) the equilibrium constants between the metal and substrate and the metal and enzyme have been determined independently. Since the kinetic constant K_a is a function of two successive association constants, it is necessary to determine the K_a for both the metal ion and the substrate. If this is not possible, additional independent evidence is necessary.

In the case of peptidases the independent evidence consisted of the lag period in activation, the absence of such a period for carnosinase leaves only kinetic evidence and determinations of metal substrate interaction. Of the metal ions activating carnosinase, Mn^{++} shows the least tendency to associate with the substrate. However, if the activity is plotted in a Lineweaver-Burk plot against the reciprocal concentration of the Mn-substrate 1:1 complex, the apparent Michaelis constant at 40° is approximately 6×10^{-5} moles/l which is a magnitude not impossible for enzymes. This means that there is at present no evidence about the path of the enzymic reaction. Equations 7 and 8 represent by no means the only alternatives compatible with the kinetic data; other mechanisms are possible (34).

In the case of Cd^{++} activation we can only argue that if the metal in the ternary complex is attached to both the substrate and the enzyme, it is not necessary that the mechanism is represented by one pathway. On the contrary, several alternatives are possible, leading to the same symmetrical ternary complex, the contribution by each being determined by the individual association constants for each step. The relative importance of the different pathways can vary with different metals and different substrates leading to differences in kinetic behaviour for the same enzyme.

If we reconsider the Hellerman–Smith hypothesis in the light of the results from studies with carnosinase, we can say that the role of the metal ion, when it is carefully separated from the stabilization effects, is that of a coenzyme. The very strong influence from metal-ion stabilization makes it necessary to reinvestigate the claimed apparent metal-ion activations and metal-ion specificities of the enzymes hitherto classified as metal-ion activated peptidases. The combined selectivity of stabilization and activation can mostly be explained by the secondary factors influencing the concentration of free metal ions in solution. This shows that a high specificity is not a

necessary characteristic of this group of enzymes and may be due to experimental difficulties. The lack of a lag period makes the use of this phenomenon as an argument for different possible mechanisms invalid. As impure carnosinase preparations do show a lag period, the results suggest that the lag period for metal peptidases may be an artifact without any connection with the mechanism of the enzyme action.

The studies of the metal-substrate interaction give no support to the proposed chelate formation between the substrate and the metalion; nor can it be demonstrated that an electron pull of the type described in the Hellerman–Smith hypothesis can exist. On the contrary, the evidence is against such an interaction. If we then compare the determined interaction between the metal ion and the substrate, and the region of concentration of metal-ion activation, regardless of the possible path of the reaction, it is evident that the strong interaction of the substrate with the enzyme must be determined in the main part by direct interaction. On the other hand, the change in the pH-activity curves for different substrates supports the part of the Smith–Hellerman hypothesis where a direct interaction between the substrate and the metal ion is proposed. The evidence available makes it more plausible that the metal ion furnishes one point of interaction rather than acts through formation of a chelate. This is in agreement with the discovered relatively broad specificity of metal-ion activation. This would necessitate a modification of the Smith–Hellerman hypothesis in a fundamental way and it seems to be advisable before any such attempt to reinvestigate some other peptidase of this category, in order to determine to what extent the behaviour of carnosinase can be considered to be representative of the whole group of enzymes. Studies in this direction are in progress.

SUMMARY

The aim of this investigation was to study the nature of the influence of metal ions on the catalytic action of peptidases. The main effort was directed to the elucidation of the mechanism of activation. The investigation revealed, however, that also the stability of the enzyme was dependent on the metal-ion concentration. The difference between these two functions of the metal ion may often be of a purely operational nature, since, if the metal ion is a necessary part of the particular protein conformation which leads to the formation of an active enzymic site, then the effect of the metal ions can equally well be called activation as stabilization.

In the case of carnosinase, due to fortunate circumstances, these two functions could be shown to be independent of each other and studied separately. However, the measured apparent effect of the metal ions in most circumstances is a combination of both functions.

The investigations summarized here consisted of studies of metal-ion activation (22), metal-ion stabilization (19), and the interaction of the metal ions with the substrate (25, 26). The main aspects and more important conclusions of this work are listed below.

1. It has been shown that both the activity and stability of purified carnosinase is a function of the concentration of certain metal ions.

2. Both functions are independent and in the case of activation a formal analogy exists between the role of the metal ion and that of a coenzyme.

3. The activation kinetics show that the activation at low temperatures follows a simple mass-law equation which indicates a strict 1:1 stoichiometry for each active site.

4. The kinetic constants of several Mn^{++}-activated enzymes have been compared; the differences in the values of the constants are surprisingly small.

5. At higher temperatures the data for Mn^{++} activation deviate from the curve predicted by the 1:1 stochiometry. This deviation is shown to be due to the stabilization of the enzyme by Mn^{++} ions.

6. The metal-ion-free enzyme can be activated by several divalent ions; the relative efficiency is described by

$$Cd^{++} > Mn^{++} > Zn^{++} > Co^{++}.$$

Of other metal ions tested, Be^{++} and Fe^{++} showed strong inhibiting properties.

7. The metal-ion-free carnosinase is extremely unstable. The kinetics of the irreversible inactivation has been studied and a mechanism for the process proposed.

8. The enzyme is stabilized by a number of divalent ions. The efficiency of stabilization is given by the following order:

$$Mn^{++} > Ca^{++} > Mg^{++} \simeq Cd^{++} > Zn^{++}.$$

9. It was shown that Ca^{++} as a stabilizer does not inhibit Mn-activation competitively, which is explained by the existence of different sites for activation and stabilization.

10. The interaction of the activating metal ions with the substrate have been studied by electron spin resonance, potentiometric titration and metal-indicator methods.

11. The influence of metal ions on the peptide bond has been studied by infra-red spectroscopy. The results indicate that an electron dislocation takes place in Cu^{++} but not in Ni^{++} or Zn^{++} chelates of dipeptides.

12. The magnitude of the formation constants and the results from infra-red studies indicate that chelate formation between the metal and the substrate is very improbable.

13. The differences in pH dependence of metal-ion activation with different metal ions and different substrates indicates that the metal-substrate interaction takes place during the enzymic catalysis. The metal-substrate interaction is proposed to take place at the imidazole ring.

14. The apparent specificity of carnosinase under different conditions is a function of both activation and stabilization. The intrinsic, relatively broad, metal-ion specificity is considered to support the assignment of one binding site on the substrate for interaction with metal ions.

15. The lag period in metal-ion activation has been shown to be absent in the purified carnosinase. It is proposed that the lag period in tissue extracts is connected with the presence of partially denaturated enzyme and its nature is irrelevant to the process of metal-ion activation of the enzyme.

16. The Smith-Hellerman hypothesis of metal-ion activation of peptidases is discussed on the basis of the experimental material presented.

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